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Application of Multilocus Enzyme Gel Electrophoresis to *Haemophilus influenzae*

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Multilocus enzyme electrophoresis was adapted to the study of *Haemophilus influenzae*. Protein extracts from sonicated whole bacteria were subjected to starch gel electrophoresis. After staining with substrates, the position of each isoenzyme (electromorph) was registered. Each isolate was assigned an electrophoretic type (ET) by the combination of electromorphs for the enzymes stained. Twenty-seven enzymes were tested; 12 were expressed in *H. influenzae*. Six enzymes were selected for subsequent study: malate dehydrogenase (MDH), phenylalanylleucine peptidase (PE2), 6-phosphogluconate dehydrogenase (6PG), adenylate kinase (AK), glucose 6-phosphate dehydrogenase (G6P), and phosphoglucose isomerase (PGI). They were polymorphic and occurred in all isolates. Six electromorphs were found for PE2, G6P, and PGI, five for MDH, four for 6PG, and three for AK. PE2, G6P, and PGI contributed most of the ET resolution (48 of 49 ETs). Multilocus enzyme electrophoresis showed several advantages over previous typing techniques. (i) An ET could be assigned to both typable and nontypable (NT) isolates. (ii) The technique was powerful in resolving differences among isolates. The 94 isolates comprised 49 ETs, five biotypes, and six capsular types and NT isolates. (iii) Strains known to be related expressed the same ET, e.g., RAB b^+ and b^-, ET12; Ma a^+ and a^-, ET1. ET variability among type b isolates was low; 26 of 28 clinical isolates expressed ET14; 2 of 28 expressed ET13 and ET15, differing from ET14 by one electromorph each. In contrast, the 47 NT isolates comprised 38 different ETs. No ETs were shared between non-type b capsulated strains and type b or NT strains. Interestingly, five NT isolates expressed the same ET as type b strains. (iv) Strains of the same capsular type but different biotypes expressed different ETs. ET determinations will thus be useful in studying the epidemiology and evolution of *H. influenzae*.

Multilocus enzyme electrophoresis was first used to study genetic diversity in eucaryotes (8, 9, 14). Recently, it has been used to analyze genetic variation in natural populations of bacteria (6, 7, 19, 23, 25, 29). With this technique, genetically controlled variants of enzymes (allozymes) differing in net electrostatic charge as a result of one or more amino acid substitutions are separated in a starch gel. The allozymes are identified after histochemical staining with specific substrates by visually comparing the relative mobility of the enzyme variants side by side on the gel. The electrophoretic type (ET) of an isolate is defined by the combination of electromorphs for the enzymes tested.

By using a relatively large number of enzymes, a positive correlation between estimates of relatedness obtained by enzyme electrophoresis and by DNA hybridization of whole chromosomal DNA has been demonstrated in higher organisms (31) and in bacteria (5, 25), showing that multilocus enzyme electrophoresis gives a representative measure of relatedness between individuals. Accordingly, *Escherichia coli* strains of the same ET were also closely related in other characteristics, e.g., O:K:H serotype (6).

Several properties have been used to identify isolates of *Haemophilus influenzae*. For routine epidemiology, capsular type and biotype are accessible. Six capsular types can be distinguished by typing with specific antisera (26). Nontypable (NT) strains do not react with these typing antisera. The biotyping scheme of Kilian (12) differentiates seven groups by known metabolic markers. Recently, patterns of lipopolysaccharide and outer membrane proteins (OMPs) have been shown to discriminate *H. influenzae* isolates of identical biotype and capsular type (3, 4). Eleven lipopolysaccharide subtypes and at least 21 OMP subtypes were shown in type b strains (3, 11), and high variability was observed among NT strains (4).

Our search for an additional typing technique to characterize *H. influenzae* isolates was prompted by an interest in infections caused by NT *H. influenzae* strains (27; O. Porras, H. Dillon, B. Gray, and C. Svanborg-Edén, Pediatr. Infect. Dis., in press). Since the type b capsule is associated with systemic infections, the previous identification schemes have been designed to further classify the encapsulated isolates. The present study describes the successful use of multilocus enzyme electrophoresis in both encapsulated and NT isolates and how the results correlate with those of other typing techniques.

(Selected aspects of this study were presented in June 1985 at the Federation of European Microbiology Societies meeting [Molecular biology of microbial pathogenesis: role of protein carbohydrate interaction, S. Normark and D. Lark, ed., in press].)

MATERIALS AND METHODS

Bacteria. A total of 94 *H. influenzae* isolates were tested (Table 1). Nineteen were obtained from the National Collection of Type Cultures (NCTC), Public Health Laboratory, London, and from the Culture Collection of the University of Göteborg (CCUG), Göteborg, and were kept lyophilized

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TABLE 1. *H. influenzae* strains studied[a]

Isolation site[a]	No. of strains							
	Total	Capsular type			Biotype			
		b	Non-b	NT[b]	1	2	3	Other
Blood	14	10	1	3	10	4	0	0
CSF	13	12	1	0	13	0	0	0
MEF	9	0	0	9	1	6	1	1
NPX	35	3	0	32	4	20	11	0
Other	4	0	2	2	2	2		0
Unknown	19	4	11	4	10	2	1	6
Total	94	29	15	50	40	34	13	7

[a] CSF, Cerebrospinal fluid; MEF, middle ear fluid; NPX, nasopharynx.
[b] NT strains included three noncapsulated mutants from strains of capsular types a, b, and f.

until used. Of them, 15 were encapsulated (type a: CCUG 6881, NCTC 8465, NCTC 8466; type b: strain RAB CCUG 12769; type c: CCUG 4852, NCTC 8469; type d: CCUG 6878, CCUG 15517; type e: NCTC 8472, CCUG 15518, Montenegro, CCUG 15522, 386/66; type f: NCTC 8473, CCUG 15435, NCTC 7918), three were noncapsulated mutants of encapsulated strains type a, b, and f (CCUG 187, CCUG 12769, and CCUG 12771). The remaining 75 strains were isolated from patients with different types of infections in Göteborg, Sweden.

The clinical isolates were confirmed as *H. influenzae* by their growth requirement for both NAD and hematin. At isolation each strain was designated type b or NT by using the Phadebact Haemophilus Test (Pharmacia, Uppsala, Sweden) and kept frozen at −70°C in 1 ml of broth. The capsular types of the reference strains were determined by the suppliers.

For biotyping and protein extract preparations, the bacteria were thawed, transferred to hematin plates, and grown overnight at 37°C in 5% CO_2. The biotype was determined by the ability of each strain to produce urease, indole, and ornithine decarboxylase as described by Kilian et al. (13).

The capsular type and biotype of the *H. influenzae* strains is shown in Table 1. All the capsular types (a through f) and biotypes 1 through 4 and 7 were represented. The combinations of capsular type and biotype were as follows: a:1, b:1, b:2, c:2, d:4, e:4, e:3, f:1, f:2, NT:1, NT:2, NT:3, and NT:7. Biotypes 1 and 2 were expressed by 43 and 36% of the strains, respectively. The rest of the isolates were biotype 3 (14%), biotype 4 (6%), or biotype 7 (1%).

Of the capsular type b strains, 93% were biotype 1. Among the NT strains, 62% were biotype 2, 26% were biotype 3 and 11% were biotype 1. Equal numbers of non-type b capsulated strains (33%) expressed biotypes 1, 2, and 4. One type e strain was biotype 3 (Table 1).

Bacterial protein extract preparation. Protein extracts were prepared from organisms grown in a medium developed by E. Falsen and A. Malmborg, University of Göteborg. The medium contained 23 g of special peptone (Oxoid Ltd., Hampshire, U.K.), 1 g of soluble starch (Difco Laboratories), and 5 g of NaCl in 1 liter of deionized water (pH 7.3). The components were solubilized by heating and autoclaved at 120°C for 20 min. Thereafter, 30 ml of sterile yeast autolysate, 15 ml of a 0.2% hemin solution, 15 ml of sterile IsoVitaLex, and 0.05% sterile glucose were added. After overnight growth in 500-ml flasks containing 100 ml of broth at 37°C with shaking, the cultures were centrifuged at 8,670 × *g* for 10 min at 5°C (J2-21 rotor JA 10; Beckman Instruments, Palo Alto, Calif.). The pellet was suspended in 2 ml of 0.01 M Tris–0.001 M EDTA (pH 6.8), transferred to 11-ml polycarbonate tubes (Nunc, Kamstrup, Denmark), sonicated for 1 min with cooling (100-W Ultrasonic Desintegrator; MSE, London, U.K.), and centrifuged at 2,830 × *g* for 30 min at 5°C. The supernatants were transferred to 10-ml polystyrene tubes (Nunc) and stored at −70°C until used.

Enzyme starch gel electrophoresis. Gels containing 11.4% starch (Connaught Laboratories, Toronto, Canada) were prepared, and the protein extracts were subjected to horizontal starch gel electrophoresis as described previously for *E. coli* and *Shigella* (29; D. Caugant, Ph.D. thesis, University of Göteborg, Göteborg, Sweden, 1983), and the following 27 enzymes were tested: malate dehydrogenase (MDH; EC 1.1.1.37), phenylalanylleucine peptidase (PE2; EC 3.4.11.-), 6-phosphogluconate dehydrogenase (6PG; EC 1.1.1.44), adenylate kinase (AK; EC 2.7.4.3), glucose 6-phosphate dehydrogenase (G6P; EC 1.1.1.49), phosphoglucose isomerase (PGI; EC 5.3.1.9), fumarate hydratase (FH; EC 4.2.1.2), phosphoglucomutase (PGM; EC 2.7.5.1), glutamic oxaloacetic transaminase (GOT; EC 2.6.1.1), glyceraldehyde 3-phosphate dehydrogenase (G3P; EC 1.2.1.12), hexokinase (HEX; EC 2.7.1.1), indophenyl oxidase (IPO; EC 1.15.1.1), acid phosphatase (EC 3.1.3.2), aconitate hydratase (EC 4.2.1.3), alanine dehydrogenase (EC 1.4.1.1), alcohol dehydrogenase (EC 1.1.1.1), alkaline phosphatase (EC 3.1.3.1), catalase (EC 1.11.1.6), esterase (EC 3.1.1.1), glutamate dehydrogenase (EC 1.4.1.4), isocit-

TABLE 2. Horizontal starch gel electrophoresis conditions and electromorphs for the enzymes detected in *H. influenzae*

Enzyme	V	Buffer system	MD[a] (cm)	No. of strains	No. of EMs[b]	EM types					
						1	2	3	4	5	6
MDH	130	1	9	94	5	F^{++}	F^{+}	F	M	S	
PE2	130	1	8	94	6	M	M^{-}	S	S1	S2	S3
6PG	130	1	9	94	4	F^{++}	F^{+}	F	M		
AK	130	1	8	94	3	F	M	S			
G6P	250	1	10	93	6	F^{+}	F	M	S	S1	S2
PGI	150	2	10	94	6	F^{+}	F	M	S^{+}	S	S1
FH	150	2	10	65	5	F	M	S^{+}	S	S^{-}	
PGM	130	1	9	43	6	F^{+}	F	M^{+}	M	S	S1
G3P	250	1	10	18	2	F	M				
HEX	250	1	10	9	5	F^{+}	F	M^{-}	S	S1	
GOT	250	4	10	10	2	F	M				
IPO	130	1	9	3	2	F	M				

[a] MD, Migration of dye.
[b] EMs, Electromorphs.

rate dehydrogenase (EC 1.1.1.42), lactate dehydrogenase (EC 1.1.1.28), leucylglycylglycine peptidase (EC 3.4.11.-), leucine aminopeptidase (EC 3.4.1.1), L-leucine dehydrogenase (EC 1.4.3.2), mannose 6-phosphate isomerase (EC 5.3.1.8), and sorbitol dehydrogenase (EC 1.1.1.14).

Four buffer systems (BS) were used: Tris-citrate (pH 8.0)/Tris-citrate (pH 8.0) (BS1), Tris-citrate (pH 6.3)/Tris-citrate (pH 6.7) (BS2), phosphate (pH 7.0)/phosphate (pH 6.7) (BS3), and borate (pH 8.2)/Tris-citrate (pH 8.7) (BS4). The electrophoretic mobility of the allozymes was determined by visually comparing their relative migration side by side on a gel (D. Caugant Ph.D. thesis).

After electrophoresis the gels were sliced, stained with specific substrates as described by Selander (29) and Caugant (Ph.D. thesis), read, fixed in acetic acid-methanol-water (1:5:5), and photographed.

Histochemical stains. The staining recipes for the enzymes expressed by *H. influenzae* (see Table 2) are given below. Remaining recipes are available (D. Caugant, Ph.D. thesis). Amounts given are for one gel. The composition of each stain was as follows: (i) MDH: 40 ml of 0.2 M Tris hydrochloride (pH 8.0), 6 ml of 2.0 M malic acid (stock solution: 268.2 g of malic acid and 160.0 g of NaOH dissolved in 1 liter of deionized water, pH adjusted to 7.0 with NaOH), 25 mg of NAD, 25 mg of Nitro Blue Tetrazolium (NBT), and 6 mg of phenylmethylsulfonyl fluoride (PMS). (ii) 6PG: 10 ml of 0.2 M Tris hydrochloride (pH 8.0), 7 ml of 0.1 M $MgCl_2$, 20 mg of barium 6-phosphogluconic acid, 1 mg of NADP, 1 mg of PMS, 3 mg of MTT (sufficient to stain three gel slices). (iii) AK: 25 ml of 0.2 M Tris hydrochloride (pH 8.0), 25 mg of ADP, 900 mg of glucose, 5 mg of HEX, 1.1 ml of 0.1 M $MgCl_2$, 17.5 mg of NADP, 6 mg of PMS, and 5 mg of MTT. In a separate flask, 500 mg of agar was boiled in 25 ml of water. When the temperature had decreased to 60°C, the agar was mixed with the staining solution and the gel was overlaid with the solution. (iv) PE2: 10 mg of peroxidase, 10 mg of *o*-dianisidine dihydrochloride, 20 mg of L-phenylalanyl-L-leucine, 10 mg of Bothrops atrox snake venom in 25 ml of 0.2 M Tris hydrochloride (pH 8.0) with 0.5 ml of 0.25 M $MnCl_2$. In a separate flask, 500 mg of agar was boiled with 25 ml of 0.2 M Tris hydrochloride (pH 8.0), and the procedure described above for AK was used. (v) G6P: 4 ml of 0.5 M Tris hydrochloride (pH 7.2), 30 ml of deionized water, 230 mg of glucose 6-phosphate, 25 mg of sodium cyanate, 211 mg of NADP, 7.5 mg of NBT, and 2 mg of PMS. (vi) PGI: 25 ml of 0.2 M Tris hydrochloride (pH 8.0), 10 mg of fructose 6-phosphate, 0.3 ml of 0.1 M $MgCl_2$, 0.3 ml of a 10-U/ml G6P water-glycerol (1:1) solution, 6 mg of NADP, 1 mg of PMS, and 10 mg of MTT. The gel was overlaid with the staining solution mixed with agar as described for PE2. (vii) FH: 50 ml of 0.2 M Tris hydrochloride (pH 8.0), 50 mg of fumaric acid, 2.5 ml of a 10-U/ml MDH solution, 20 mg of NAD, and 5 mg of NBT. When ready to stain, 0.5 ml of 1% PMS was added. (viii) PGM: 25 ml of deionized water, 5 ml of 0.2 M Tris hydrochloride (pH 8.0), 5 ml of 0.05 M disodium α-D-glucose-1-phosphate, 5 ml of 5×10^{-4} M dipotassium α-D-glucose-1,6-diphosphate, 5 ml of 0.1 M $MgCl_2$, 4 ml of G6P (see above for PGI), 5 mg of NADP, 5 mg of MTT, and 5 mg of PMS. (ix) G3P: 12 ml of 0.5 M Tris hydrochloride (pH 7.1), 38 ml of deionized water, 225 mg of fructose 1,6-diphosphate, 50 U of aldolase, 75 mg of sodium arsenate, 25 mg of NAD, 15 mg of NBT, and 10 mg of PMS. (x) HEX: 10 ml of 0.2 M Tris hydrochloride (pH 8.0), 30 ml of deionized water, 10 ml of 0.1 M $MgCl_2$, 75 mg of D-glucose, 60 mg of ATP, 5 mg of NADP, and 0.8 ml of a 10-U/ml G6P water-glycerol (1:1) solution. When ready to stain, 4 mg of PMS was added. (xi) GOT: 0.5 mg of pyridoxal 5-phosphate, 50 mg of L-aspartic acid, 100 mg of α-ketoglutaric acid, and 100 mg of fast blue BB salt dissolved in 50 ml of 0.2 M Tris hydrochloride (pH 8.0). (xii) IPO: 40 ml of 0.2 M Tris hydrochloride (pH 8.0) and 1 ml of 0.1 M $MgCl_2$. When ready

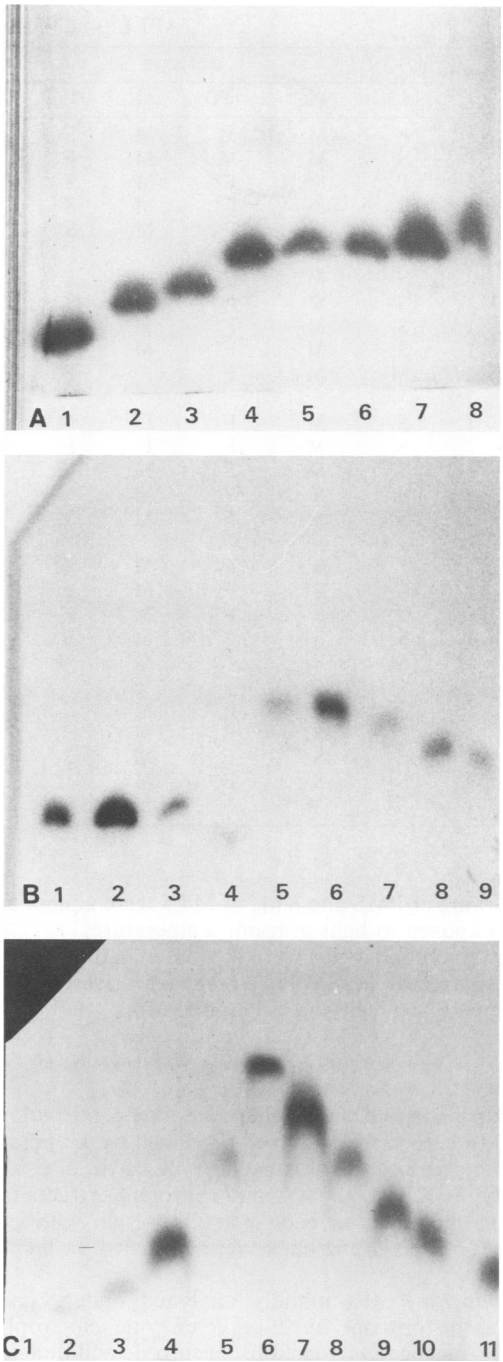

FIG. 1. Electromorphs of *H. influenzae* and *E. coli*. (A) G6P. Lanes 1 through 4, *E. coli* 553, Gina 1, 548, and 556; lanes 5 through 9, *H. influenzae* electromorphs F^+, F, M, S, and S1. (B) PGI. Lanes 1 through 4, *H. influenzae* electromorphs S1, S, M, and F; lanes 5 through 8, *E. coli* 598, ABU 83, Gina 1, and 631. (C) MDH. Lanes 1 through 6, *E. coli* 312, 129, 529, Gina 1, 18, and 327; lanes 7 through 11, *H. influenzae* electromorphs F^{++}, F^+, F, M, and S.

TABLE 3. ETs identified in 94 isolates of *H. influenzae*

ET no.	No. of strains	Isozymes						ET no.	No. of strains	Isozymes					
		MDH	PE2	6PG	AK	G6P	PGI			MDH	PE2	6PG	AK	G6P	PGI
1	3	F	M	M	M	F	F	26	1	M	S	F^+	M	F	M
2	1	S	M	M	M	F^+	F	27	2	F	M	F^+	M	S1	S1
3	2	F^{++}	S	F^+	M	S	S1	28	2	F^+	S	F^{++}	M	M	M
4	1	S	S	F^+	M	S	S	29	1	S	S1	F^+	M	S1	S1
5	1	S	S	F^+	M	S	F^+	30	1	S	S2	F^+	M	S1	S1
6	2	S	M^-	F	M	F	S1	31	1	F^+	S3	F^{++}	S	S	S
7	2	S	S	F^+	M	S	F	32	1	S	M	F^+	M	S1	F
8	1	S	M	F^+	M	M	S1	33	1	S	M	F^+	M	F	S1
9	1	S	M	F^+	M	M	S^+	34	1	F	M	F	M	S	S1
10	2	M	M	F	F	F	M	35	4	S	S1	F^+	M	M	F
11	1	S	M	F	F	M^-	N	36	1	S	S1	F^+	M	M	M
12	2	S	M	F	F	F	M	37	1	S	S1	F^+	M	M	S1
13	3	S	S	F^+	M	M	F	38	2	S	M^-	F^+	M	M	F
14	28	S	S	F^+	M	M	S	39	1	F^+	S	F^+	M	S	S1
15	2	S	M	F^+	M	M	S	40	1	S	S	F^+	M	M	S1
16	1	F	S	F	M	S1	S1	41	1	M	S1	F^+	M	S	S1
17	1	S	S	F^+	M	F	M	42	1	M	S	F^+	M	S	M
18	1	S	S	F^+	M	F	F	43	2	F^+	S	F^+	M	S	M
19	1	S	S1	F^+	M	S	S	44	1	S	S	F^+	M	S	S1
20	1	M	S	F^+	M	M	M	45	1	F	M^-	F	M	S1	S1
21	1	S	S2	F^+	M	S	S1	46	1	M	M	F^+	F	M	S1
22	1	F^+	S2	F	M	S1	S	47	1	M	S	F	M	F^+	S1
23	1	S	S	F	M	S	F	48	1	S	S3	F^+	M	M	S1
24	2	S	S	F^+	M	M	M	49	1	S	M	F^+	S	S2	M
25	1	S	M^-	F^+	M	F	S1								

to stain, 5 mg of PMS and 8 mg of MTT were added, and the gel was exposed to light at room temperature.

Except for IPO, all gels were incubated in the dark at 37°C until staining was complete. All reagents were purchased from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Standardization of the technique. Seventeen isolates were selected to screen for enzymes produced by *H. influenzae*. They included isolates of capsular types a, b, c, e, and NT and biotypes 1, 2, 3, and 4. Control protein extracts from *E. coli* were included on each gel. Only gels with positive staining for *E. coli* enzymes were regarded as technically acceptable.

Each enzyme was initially analyzed under conditions shown to be optimal for *E. coli* enzyme electrophoresis (Table 2). If negative reactions occurred, additional buffer systems (BS1 through BS4) were tried. BS1 and BS2 worked for all positive enzymes except GOT, which required BS4. BS3 did not resolve any additional enzyme activities.

Fifteen of the 27 enzymes assayed were not detected among the 17 *H. influenzae* strains regardless of assay conditions (acid phosphatase, aconitate hydratase, alanine dehydrogenase, alkaline phosphatase, alcohol dehydrogenase, catalase, esterase, glutamate dehydrogenase, isocitrate dehydrogenase, lactate dehydrogenase, leucylglycylglycine peptidase, leucine aminopeptidase, L-leucine dehydrogenase, mannose 6-phosphate isomerase, sorbitol dehydrogenase).

The electromorphs of enzymes most frequently detected among the 94 isolates and the assay conditions are shown in Table 2. MDH, PE2, 6PG, AK, G6P, and PGI occurred in all isolates, except for a null in G6P. Other enzymes were less frequent; FH occurred in 65, PGM in 43, G3P in 18, and HEX in 9 of 94 strains. All enzymes were polymorphic and migrated toward the anode. Six electromorphs were detected for enzymes PE2, G6P, PGI, and PGM; five for MDH, FH, and HEX; four for 6PG; three for AK; and two for G3P, IPO, and GOT. The mobility was graded as variants of fast (F^{++}, F^+, F), medium (M, M^-), or slow (S^+, S, S1, S2, S3). Examples are shown in Fig. 1.

Reproducibility. The reproducibility of ET determinations was assessed by repeated runs of protein extracts from 23 isolates, all encoded, with staining for each of the six standard enzymes. The same mobility was assigned on 392 of 404 occasions. The variant mobilities were one step away from those expressed in the majority of runs. In a series of three to five repeats for one allozyme, the aberrant electromorph never registered more than once.

Comparison of electromorphs. *E. coli* and *H. influenzae* differed both in the repertoire of enzymes expressed and in their mobilities. *E. coli* expressed 27 enzymes, compared with 12 in *H. influenzae*. None of the electromorphs of *H. influenzae* and *E. coli* were found to comigrate. *E. coli* expressed four electromorphs, A through D (Fig. 1A) for G6P. The six electromorphs of *H. influenzae* ran faster than those of *E. coli* B (strains BRL6-1 and Gina) but slower than those of *E. coli* A. For PGI, all *H. influenzae* electromorphs ran slower than *E. coli* electromorphs (Fig. 1b). For MDH, the *H. influenzae* electromorphs had a mobility between those of *E. coli* M and *E. coli* S (Fig. 1c). For PE2, the six *H. influenzae* electromorphs ran slower than those of *E. coli* M. All the 6PG *H. influenzae* electromorphs were faster than those of *E. coli* F strain Gina 1 for AK and slower than the *E. coli* F^{++} enzyme.

The nomenclature of the *H. influenzae* electromorphs was therefore selected independently of that for the *E. coli* electromorphs; they were graded 1 for the fastest and 6 for the slowest mobility.

Definition of ET. Each *H. influenzae* isolate was assigned an ET by the combination of the electrophoretic forms (electromorphs) for the enzymes tested, in the order MDH, PE2, 6PG, AK, G6P, and PGI (Table 3).

Strains expressing the same electromorphs for the six enzymes were included in one ET. Strains differing by a null were assigned to different ETs. The relationship between the number of enzymes assayed and the number of ETs discriminated is shown in Fig. 2, starting with the more polymorphic enzymes and adding the number of newly identified ETs at the introduction of each new enzyme. PE2, G6P, and PGI contributed most to the resolution of ETs, AK the least. By adding FH, two new variants of ET14 were detected (data not shown).

Distribution of ETs. A total of 49 ETs were detected among 94 *H. influenzae* isolates tested, with a mean number of 1.98 ET per strain (range, 1 to 28) (Table 3). The ETs of the largest number of isolates were 14 (28 isolates), 35 (4 isolates), 1 and 13 (3 isolates each), and 3, 6, 7, 10, 15, 24, 27, 28, 38, and 43 (2 isolates each). The remaining 36 isolates each corresponded to one ET. The genetic variability will be the topic of a separate study.

Variation in capsular type and biotype within ETs. The capsular types and biotypes of isolates classified into the same ET are shown in Table 4. ETs 1 through 11 comprised the isolates of capsular types a and c through f, ETs 12 through 15 comprised the isolates of capsular type b; and ETs 13 through 49 comprised the NT isolates. Each of ETs 1 through 11 corresponded to only one capsular type and one biotype. Type e ET7 strains, however, expressed biotypes 3 and 4. ETs 12 through 15 included the type b strains but also NT isolates and expressed biotypes 1 through 3. This was the most heterogeneous group of strains. ETs 16 through 49 mostly comprised a single NT isolate each; those including several isolates belonged to the same biotype, except the two ET24 isolates expressing biotypes 2 and 3.

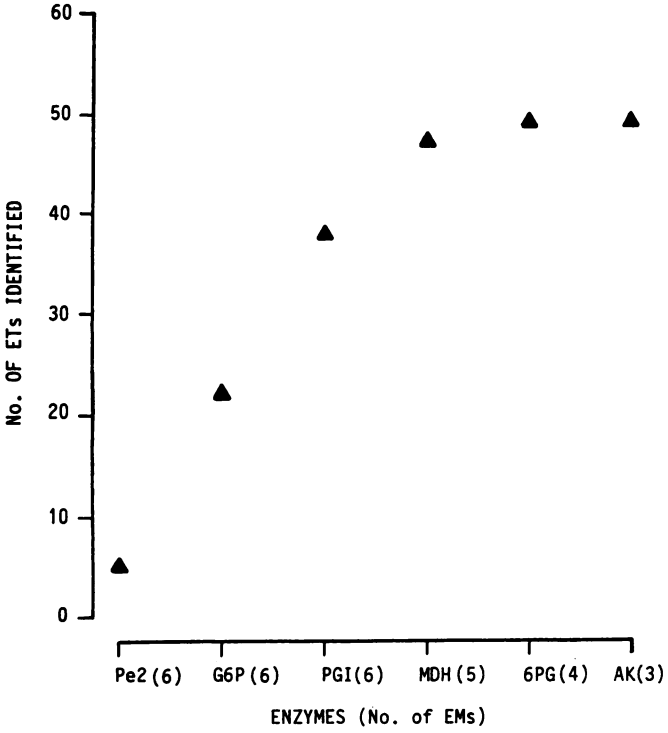

FIG. 2. Contribution to the resolution of different ETs from the enzymes PE2, G6P, PGI, MDH, 6PG, and AK. The number of electromorphs (EMs) for each enzyme is shown in parentheses. The cumulative frequency of ETs for each added enzyme is shown.

TABLE 4. Capsular type and biotype variation within ETs[a]

ET	No. of strains	Capsular type(s) (no. of strains)	Biotype(s)
1	3	a	1
3	2	c	2
6	2	e	4
7	2	e	3, 4
13	3	b (1), NT (2)	1, 2
14	28	b (26), NT (2)	1, 2, 3
15	2	b, NT	1, 2
24	2	NT	2, 3
27	2	NT	1
28	2	NT	3
35	4	NT	2
38	2	NT	3

[a] Classes represented by more than one isolate are shown.

Variation in ET within capsular type and biotype. The ETs expressed by isolates of the same capsular type, biotype or capsular type-biotype class are shown in Table 5. The type b strains expressed four ETs. Of 28 capsulated type b strains, 26 were ET14 (Table 4), one strain was ET13, and another was ET15. ET14 differed from ET13 only at enzyme PGI and from ET14 at enzyme PE2. The fourth, ET12, expressed by strain RAB, differed in five enzymes from ET13 and ET14 and in four enzymes from ET15. Twenty-three of the ET14 strains were analyzed for FH. The additional enzyme discriminated two subgroups, one comprising 21 strains and the other 2 strains.

No ETs were shared between capsulated type b and non-type b capsulated *H. influenzae* strains. In addition, no ETs were shared between the non-type b capsular types (Tables 4 and 5).

Thirty-eight ETs were detected among the 47 NT isolates, with a ratio of 0.81 ET per strain (Table 5). Two or more NT strains were ET 24, 27, 28, 35, 38, or 43 (Table 4). Five NT strains belonged to the ETs expressed by the type b strains. Two were ET13, two ET14 and one ET15. No ETs were shared between the NT and non-type b capsulated strains. The variation in ET among NT *H. influenzae* strains was significantly higher (38 of 47 strains) than among the type b strains (4 of 28 strains) ($P < 0.01$).

The ratio of ETs to isolates was similar within and between biotypes 2, 3, and 4 (Table 5). The lower ratio for biotype 1 was secondary to the dominance of type b isolates in this group.

When capsular type and biotype were analyzed as a class (Table 5), a low ratio of ETs per class (0.15) was recorded for *H. influenzae* type b biotype 1 strains. Most classes comprised one isolate and corresponded to only one ET. The three more frequent combinations among the NT isolates (NT:1, NT:2, and NT:3) showed ratios of 0.80, 0.85, and 0.85 ET per group, respectively (Table 5). The combination capsular type e-biotype 4 showed a ratio of 0.75 ET per class (Table 5). The correlation of ET, capsular type, biotype, and OMP is presented in reference 27.

Site of isolation. The variation in ET was low in blood and

TABLE 5. ET variation within capsular type and biotype in *H. influenzae*

Typing method	No. of strains	No. of ETs	ET no.	Ratio (ET/strain)
Serotype				
a	4	2	1, 2	0.50
b	29	4	12–15	0.14
c	2	1	3	0.50
d	2	2	4, 5	1.00
e	5	3	6–8	0.60
f	3	3	9–11	1.00
NC[a]	3	3	1, 10, 12	1.00
NT	47	38	13–49	0.81
Biotype				
1	41	12	1, 2, 10–15, 27, 41, 44, 47	0.29
2	34	28	3, 9, 13–16, 18–21, 23, 26, 29–37, 39, 40, 46, 48, 49	0.82
3	13	11	7, 14, 17, 18, 24, 28, 38, 42, 43, 45, 50	0.86
4	6	5	4, 5–8	0.83
7	1	1	22	1.00
Serotype:biotype				
b:1	27	4	12–15	0.15
NT:1	5	4	27, 41, 44, 46	0.80
NT:2	29	25	13–16, 19–21, 23–26, 29–37, 39, 40, 45, 47, 48	0.86
NT:3	12	9	14, 17, 18, 24, 28, 38, 42, 32, 49	0.75
e:4	4	3	6–8	0.75

[a] NC, Nonencapsulated mutants of encapsulated strains.

cerebrospinal fluid (CSF) isolates. This was secondary to the dominance of type b isolates from these sites and the low ET variability among the type b strains. The 13 CSF isolates expressed capsular type a and ET 2, 6, 14, and 15, with an ET/isolate ratio of 0.23. The 13 blood isolates expressed capsular type b and ET 14, NT and ET 35 and 36; and type f and ET11, with an ET/isolate ratio of 0.31. In contrast, the nasopharyngeal and middle-ear fluid isolates showed an ET/isolate ratio of 0.71 and 0.89, respectively, with 25 ETs among 35 of the former and eight ETs among nine of the latter isolates.

DISCUSSION

Multilocus enzyme electrophoresis was shown to be highly sensitive in discriminating among strains of *H. influenzae*. By this technique, an ET was assigned to each isolate depending on the electrophoretic mobility of known enzymes in a starch gel. Several advantages over previous routine typing techniques were observed. (i) All isolates could be typed by their electromorph combination for six enzymes, including isolates not typable with anticapsular antisera. (ii) Related strains, e.g., capsulated and capsule-negative mutants of the same strain, had the same ET. (iii) The technique discriminated well between strains known to differ by other typing techniques. For example, there was no overlap in ETs between strains of different capsular types.

The definition of ETs was based on reactivity with substrates for the enzymes MDH, PE2, 6PG, AK, G6P, and PGI. These enzymes were selected for several reasons. (i) They were polymorphic. The contribution of each enzyme to the resolution of separate ETs was roughly proportional to the number of electromorphs. Thus, PE2, G6P, PGI, and MDH resolved 48 of 49 ETs in this battery of strains. 6PG added one ET, and AK none. Indeed, multilocus enzyme electrophoresis gives a bacterial phenotype which is the sum of several variables. The weight of each variable for the total resolution will be relative to the number of variants within the variable. This is comparable, for example, to serotyping *E. coli*, for which the accuracy of strain identification is adequate with three parameters, O, K, and H, but inadequate with a single antigenic determinant (2). (ii) The six enzymes were expressed by all the strains in the present study. This reduced the potential problem with nulls, i.e., enzymes not expressed by certain strains. For example, a null for G6P occurred in one isolate which was otherwise identical to the RAB strain. These were assigned separate ETs, since G6P was expressed by the remaining 93 isolates. FH, on the other hand, was only detected in 65 of 94 strains. In ET14, 21 strains expressed one FH electromorph, two strains another electromorph, and two strains were nulls. To circumvent interpretation problems of this kind, FH and other infrequently expressed enzymes were excluded. (iii) The six enzymes gave ETs which were identical in related strains. The type b strain RAB and its mutant Sb were both ET12. The type a strain Ma and its mutant Sa were both ET1. The type f strain Shawn and its mutant were both ET10.

The definition of technical conditions suitable for *H. influenzae* enzyme electrophoresis was facilitated by the extensive standardization previously achieved with *E. coli* (1, 28, 29). The selection of enzymes could not be guided by studies on metabolic pathways in *H. influenzae*, since little such information was available. The strategy selected was therefore to screen *H. influenzae* strains for expression of a wide range of enzymes known to occur in *E. coli*. Twelve of 27 enzymes had detectable activity, suggesting substrate specificity similar to that of *E. coli* and adequate assay conditions. These enzymes may indicate preferred metabolic pathways in this species.

For *E. coli*, the 12 to 15 enzymes frequently used for multilocus enzyme electrophoresis were chosen because of

technical accessibility and genetic information rather than for a special metabolic function. The *E. coli* electromorphs are products of known chromosomal loci. The genes encoding 11 of 15 enzymes map on the chromosome of *E. coli* K-12 from positions 8 to 91 (2). All 15 enzymes were produced by plasmid-free strains.

For *H. influenzae*, there is no information on the genetic basis of enzyme synthesis. The selected enzymes were produced by all isolates. They are probably chromosomally encoded, since the same extrachromosomal element is unlikely to occur in 94 isolates from unrelated hosts. We assume that the six enzymes are part of the basic metabolic machinery of *H. influenzae*, less easily lost or gained than, for example, FH, G3P, or HEX. Recent studies of the chromosomal DNA region encoding the type b capsule (19, 21) have demonstrated that loss of capsule coincided with deletion of a chromosomal DNA fragment (9). The occurrence of identical ETs in NT and type b strains in the present study suggested that none of the enzymes was encoded by that fragment. It is interesting that strains of biotype 4 were monomorphic for MDH and AK. The number of strains in this group was small, however.

The type b strains expressed a limited number of ETs, distinct from the ETs of other capsular types and most NT strains. All but two of the clinical type b isolates belonged to ET14-biotype 1. The remaining two, ETs 13 and 15, belonged to biotype 1 and differed from ET14 by only one electromorph each. They may therefore represent variants of a common ancestor strain (for analysis of clonality, please see Porras et al. [27; Pediatr. Infect. Dis., in press]). The clinical isolates all originated in Göteborg, Sweden. Type b strains from, e.g., the United States were focused to produce ETs more distant from ET14 (27). None of the clinical type b isolates examined, the U.S. strains included, was similar to the RAB strain, which differed from ET14 by five electromorphs.

The NT isolates differed from the type b strains in showing a high ET variability in spite of having been isolated in the same geographic area. The genetic diversity of the type b and NT strains is the topic of a separate study (27). However, a small number of NT isolates expressed the same ET as type b strains. These NT isolates may represent spontaneous b^- mutants. There may be a continuous renewal of the NT population by spontaneous loss of the capsule and successive genetic rearrangement. There is no evidence at present for this hypothesis. Accepting the electromorphs as representatives of allele variants at each enzyme locus, differences in ETs may be used to evaluate the genetic relatedness of *H. influenzae* strains.

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